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Structures of the tumor necrosis factor α inducing protein Tipα: A novel virulence factor from *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori **secretes a unique virulence factor, Tipα, that enters gastric cells and both stimulates the production of the TNF-α and activates the NF-κB pathway. The structures of a truncated version of Tipα (TipαN34) in two crystal forms are presented here. Tipα adopts a novel $\beta_1\alpha_1\alpha_2\beta_2$-$\beta_3\alpha_3\alpha_4$ topology that can be described as a combination of three domains. A first region consists in a short flexible extension, a second displays a dodecin-like fold and a third is a helical bundle domain similar to the sterile alpha motif (SAM). Analysis of the oligomerisation states of TipαN34 in the crystals and in solution suggests that the disulfide bridges could hold together Tipα monomers during their secretion in the gastric environment.**

Structured summary:
MINT-7033680: *TIP alpha* (uniprotkb:B2UTN0) and *TIP alpha* (uniprotkb:B2UTN0) *bind* (MI:0407) by *cosedimentation* (MI:0027)

1. Introduction

Helicobacter pylori is a Gram-negative bacterium that colonises the human stomach. *H. pylori* is reported as the major cause of several gastric pathologies such as gastritis, peptic ulceration, stomach adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphomas [1]. Virulence factors produced by *H. pylori* have a pivotal role in chronic inflammation and increase the risk of cancer [2,3]. The contribution of major virulence factors produced by *H. pylori* to cancer risks has been characterised such as the Cytotoxin associated gene A protein (CagA), the Vacuolating toxin A (VacA) and the BabA adhesin (reviewed in [2,4]). A highly immunogenic *H. pylori* protein, Tipα (for Tumor necrosis factor α – TNF-α – inducing protein) was identified as a new virulence factor activating NF-κB and inducing the expression of TNF-α, a pro-inflammatory cytokine, produced in response to *H. pylori* infection [5–7]. Tipα also enhances the expression of different chemokines, upregulates Bcl-2 gene and downregulates p53 gene, thereby contributing to cancer establishment [8,9]. Tipα is present in all *H. pylori* genomes sequenced and has been detected in numerous clinical isolates from patients affected by gastritis, gastroduodenal ulcers and gastric cancers [6]. The mode of action of Tipα remains elusive but the protein has been shown to enter gastric cells and possibly penetrate the nucleus [10]. Tipα sequence displays no similarity to any known protein but a conserved motif has been proposed between Tipα and penicillin binding protein (PBP) suggesting that the protein might adopt a similar fold [11]. The protein is secreted in the extracellular medium [5,10,12,13] and forms homodimers of 38 kDa held together by inter-subunits disulfide bridges involving C25 and C27 [6]. *In vitro*, recombinant Tipα can bind both ss- and dsDNA [14]. A fragment of Tipα starting at residue 28 (delTipα) showed a considerably reduced ability to induce TNF-α gene expression, NF-κB activation [6] and to enter gastric cells [10] suggesting an important role for the disulfide bridges.

We describe here the crystal structures of a truncated form of Tipα (TipαN34) in two different crystal forms obtained at pH 4 and pH 8.5. These structures show that Tipα adopts a novel fold, consisting of an α–β sandwich combined with a helical bundle domain. Structural comparison and analysis provides insight into regions that may be involved in protein–protein interactions within the gastric cells. Gel filtration analysis of the oligomeric state of Tipα and TipαN34 at different pHs suggests a role for the disulfide bridges during toxin secretion.

Abbreviations: MALT, mucosa-associated lymphoid tissue; PBP, penicillin binding protein; TEV, tobacco etch virus; SAD, single anomalous dispersion

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2. Materials and methods

A DNA fragment corresponding to the secreted portion of Tipα (starting at M21) was amplified from *H. pylori* genomic DNA and cloned into pET151/D- topo vector (pET-Tipα). The protein was expressed in *Escherichia coli* and purified with a His-trap column (GE Healthcare) equilibrated with buffer A (30 mM Tris pH 8, 200 mM NaCl, 5% glycerol). Tipα was eluted with a linear gradient of imidazole from 0 to 500 mM in Buffer A. After tobacco etch virus (TEV) cleavage, the protein was further purified by Nickel affinity and size-exclusion chromatography. Seleno-methionine incorporated Tipα (SeTipα) was purified as Tipα except for NaCl concentration, raised to 500 mM in all buffers. TipαN34 and SeTipαN34 were obtained by incubating respectively Tipα and SeTipα with trypsin at a protease/protein ratio of 1:1000 (w/w) on ice for 1 h. The vapour diffusion method was used for crystallisation. Form I crystals were grown by mixing equal amounts of protein (6 mg/ml) and reservoir solution containing 100 mM Na Citrate pH 4, 15 mM ATP and from 22% to 28% of Polyethylene glycol (PEG) 4000. Crystals of SeTipαN34 grew in 100 mM Na Citrate pH 4, 14% PEG6000 and 15 mM ATP. Crystal form II was obtained by mixing 2 μl of TipαN34 with 2 μl of reservoir solution (100 mM Tris pH 8.5, 26% PEG4000, 50 mM $MgCl_2$). Data were collected at ESRF beamlines (Table 1). Native and Se-Met crystal form I diffracted to 1.9 Å and 2.0 Å, respectively, and belonged to the space group $P2_1$ with two molecules per asymmetric unit (Table 1). A highly redundant dataset was collected at the Se edge with SeTipαN34 crystals. Se sites were used to obtain experimental phases using the single anomalous dispersion (SAD) method followed by density modification using SHARP [15]. ARP-WARP [16] built an initial model that was completed manually and refined against the native data using REFMAC [17]. Crystal form II diffracted at 1.7 Å and belonged to the $P2_12_12_1$ space group with two molecules per asymmetric unit (Table 1). Crystal form II structure was solved by molecular replacement using PHASER [18] and the structure of form I as a search probe. The coordinates of Tipα crystal forms I and II structures have been deposited in the PDB, entry codes 2wcq and 2wcr, respectively. Analytical gel filtration experiments were performed by injecting 200 μg of Tipα or TipαN34 on a Superdex 75 10/300 column equilibrated in buffer A or in citrate buffer (50 mM citric acid pH 4, 200 mM NaCl). Details of the methods can be found in Supplementary data.

Table 1
Data collection, phasing and refinement statistics.

	Tip-αN34 Se-Met pH 4	Tip-α^{N34} pH 4	Tip-α^{N34} pH 8.5
Data collection statistics			
Beamline	ID14-4	ID14-4	ID29
Unit cell (Å)			
a	31.64	31.36	48.88
b	102.21	101.99	67.26
c	47.53	47.25	97.47
β	94.40	94.38	
Space group	$P2_1$	$P2_1$	$P2_12_12_1$
Wavelength (Å)	0.979	0.939	0.981
Resolution limit (Å)	43.0–2.0	50.9–1.9	27.7–1.7
Total observations	151 032 (22 287)*	93 609 (10 799)	128 160 (18 556)
Unique reflections	20 350 (2989)	22 965 (3030)	35 854 (5158)
Completeness	99.9 (99.9)	98.4 (90.4)	99.3 (99.6)
Multiplicity	7.4 (7.5)	4.1(3.6)	3.6 (3.6)
$\langle I\rangle/\langle\sigma I\rangle$	7.5 (1.8)	7.6 (2.2)	10.8 (1.9)
R_{merge} (%)[b]	7.4 (40.3)	6.0 (32.5)	4.0 (40.7)
Wilson B-factor (Å^2)	24.4	25.2	26.4
Phasing statistics			
Se sites	10		
FOM (centric/acentric)	0.13/0.41		
Anomalous phasing power	1.74		
Refinement statistics			
R_{cryst}		19.6 (27.7)	18.2 (29.3)
R_{free}		23.1 (37.7)	21.9 (34.7)
RMS bonds		0.015	0.017
RMS angles		1.482	1.51
Ramachandran outliers		Ile52B	Ser49A
B-factors			
Overall		28.0	17.0
Protein atoms		27.6	15.6
Water atoms		35.0	26.3

$R_{cryst} = (\sum||F_{obs} - F_{calc}||)/(\sum||F_{obs}||)$.
* Values in parentheses refer to the highest resolution shell.
[b] $R_{merge} = \sum|I - \langle I\rangle|/|\sum\langle I\rangle|$.

3. Results and discussion

3.1. Crystal structure of TipαN34 form I

The structure of TipαN34 monomer consists of an elongated, bean-shaped α–β sandwich combined to a helical bundle domain (Fig. 1A and B). It shows an original $\beta_1\alpha_1\alpha_2\beta_2\beta_3\alpha_3\alpha_4$ topology completed by a short 3_{10} helix at the N-terminal portion (Fig. 1A and B). The antiparallel β-sheet formed by β1, β2 and β3 wraps around the N-terminal half of α1. The C-terminal portion forms a four helix bundle with α2, α3 and α4, at the top of which lies a long loop connecting α1–α2 (Fig. 1B). Differences between the two subunits reside essentially at the N-terminal region, which appears more flexible with L37 to I52 displaying high B-factor values. This extension together with the bottom part of the β-sheet also mediates inter subunit contacts via hydrophobic interactions between V40, L45, Y42 and F139 from chain A with V40, M44, L45, F139 from chain B (Fig. 1C). Those contacts are reinforced by a network of hydrogen bonds between S58, H60, E137 from both subunits resulting in a head-to-head dimer related by a pseudo twofold axis (Fig. 1C and D). However, this dimer interface, burying 814 Å^2, is centred on E137–E137 interactions, contacts that would likely be unfavoured in solution at a physiological pH.

3.2. Domain organisation and structural similarity with known proteins

A DALI [19] search identified more than 500 proteins with Z-scores lower than 4.3 but none of these structures were larger than 100 residues and no homologue was identified for the entire TipαN34 structure. Structural homology analysis divides TipαN34 in three parts: a N-terminal loop, consisting of residues N34 to I52, showing no similarities to existing structures. A second domain, consisting of β-strands β1, β2, β3 and the N-terminal half of α1, is similar to dodecin proteins, in particular to *H. salinarum* dodecin (pdb 1mog, rmsd 2.2 Å, 59 residues). Dodecins are small proteins that oligomerise in dodecamers and bind to FAD [20]. TipαN34 superimposes well with dodecin monomer (Fig. 2A) but the FAD-binding residues (W36, E45 and Q55) are not conserved. The third domain consists of the helical bundle, similar to more than 100 proteins with best scores falling into three categories: (i) the nuclear abundant RNA-binding Nab2 protein (pdb 2v75, rmsd 2.7 Å, 70 residues), (ii) proteins containing the sterile alpha motif (SAM) domain and (iii) the translation initiation factor 2 alpha, aIF2 (pdb 1yz6, rmsd 4.8 Å, 90 residues) (Fig. 2B). The helical bundles of Tipα and Nab2 are similar to the PWI fold, a domain found in several nucleic acid binding proteins [21]. However, the motif involved in DNA binding in PWI-containing proteins is not conserved in Tipα nor in Nab2 [21]. More than 20 proteins containing a SAM domain were also identified as putative homologues, includ-

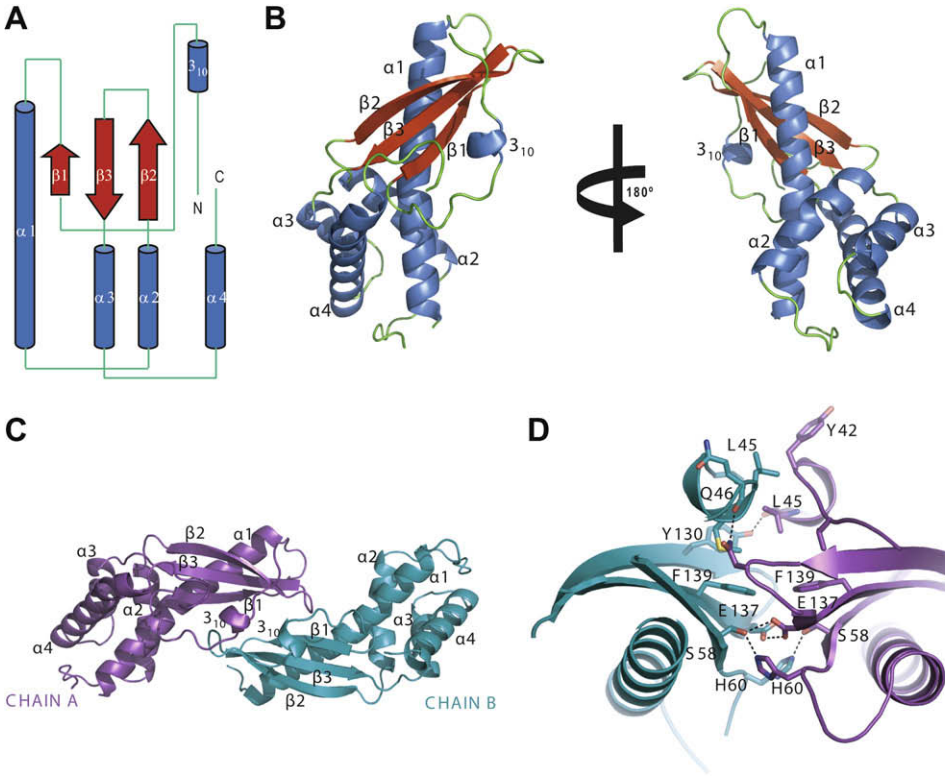

Fig. 1. Structure of TipαN34. (A) Topology diagram of TipαN34. (B) Two different views of TipαN34 structure with α-helices in blue, β-strands in red and loops in green. (C) Ribbon representation of the dimer with chain A and B colored in violet and blue, respectively. (D) Head-to-head dimer interface depicted in ribbon with side chains indicated as sticks (with carbon atoms colored depending of the subunit, nitrogen in blue, oxygen in red and sulfur in yellow).

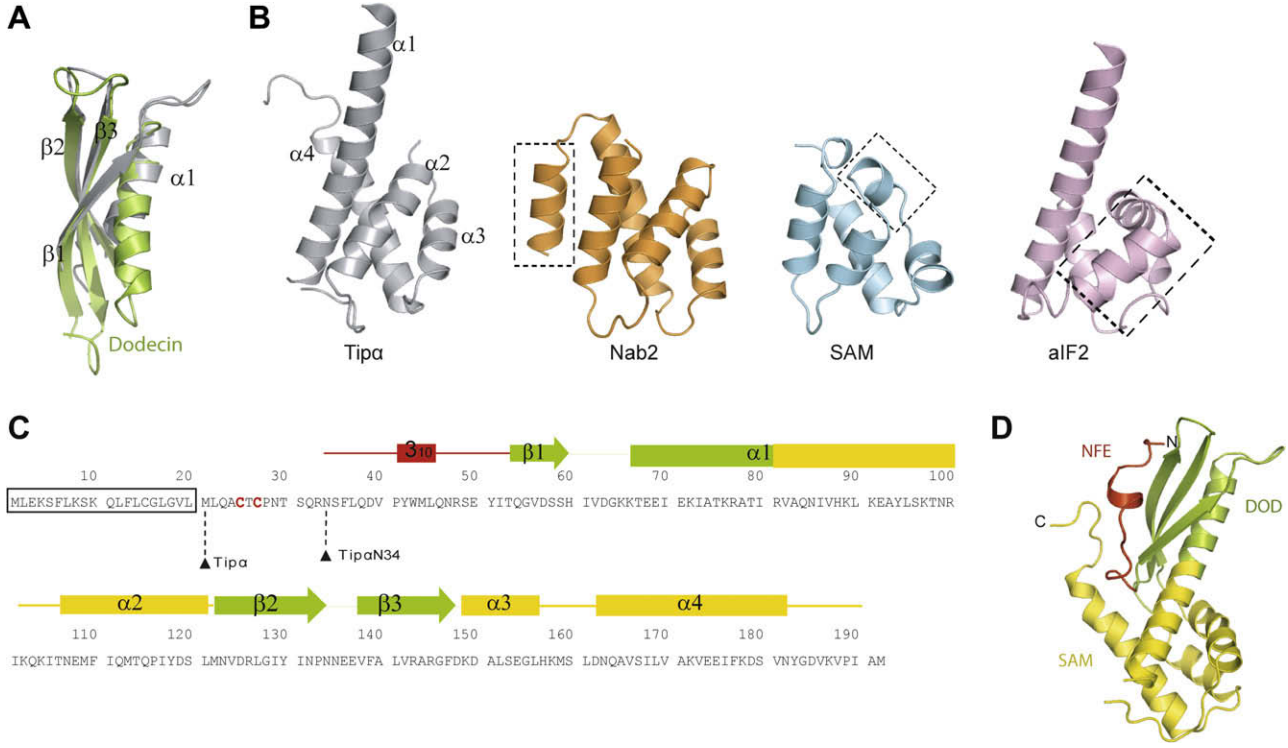

Fig. 2. Domain organisation of Tipα structure. (A) Structural superimposition of TipαN34 DOD (grey) with dodecin (green). (B) Comparison of the Tipα SAM domain (grey) with the structure of Nab2 (orange), SAM domain from polyhomeotic-proximal chromatin protein (blue) and aIF2 (pink). Areas of main divergence are indicated by dashed boxes. (C) Sequence of Tipα with secondary structures indicated above and colored according to their respective domains (NFE, red; DOD, green and SAM, yellow). The signal peptide is indicated by a black box, C25 and C27 are colored in red. Starting residues for Tipα and TipαN34 are indicated. (D) Structure of TipαN34 colored according to domain boundaries.

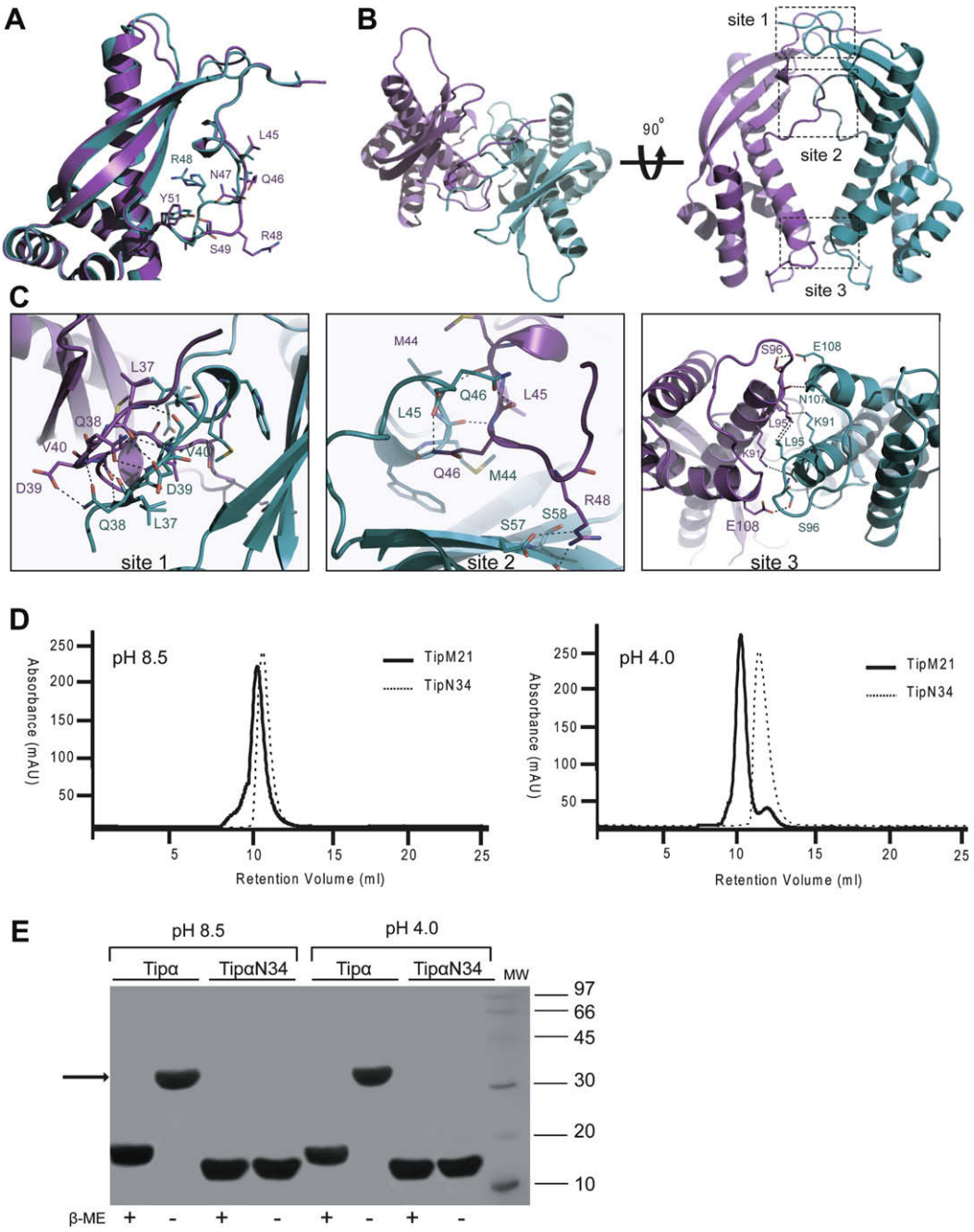

Fig. 3. Crystal form II of TipαN34. (A) Superimposition of chain A and chain B showing the different conformations of NFE loop. (B) Two views of the dimer of TipαN34 at pH 8.5 with chain A and B colored in violet and blue, respectively. Dotted boxes points to the three contact sites between subunits. (C) Close-up view of TipαN34 dimer interaction sites indicated in (A). Side chains of the residues involved are displayed in sticks. (D) Gel filtration analysis of Tipα and TipαN34 at pH 4 and pH 8.5. (E) SDS–PAGE analysis of the gel filtration experiments at pH 8.5 and pH 4 in the presence or absence of 5 mM β-mercapto-ethanol (β-ME). The arrow indicates Tipα dimer and the molecular weight markers (MW) sizes are indicated on the right in kDa.

ing polyhomeotic-proximal chromatin, sex comb, aveugle and ephA4 receptor tyrosine kinase. SAM domains are short domains found in a wide variety of eukaryotic proteins and in few bacterial ones [21,22]. The structural similarity of Tipα to SAM domain raises interesting questions since these domains can mediate protein–protein, protein–DNA and protein–RNA interactions [23]. TipαN34 also resembles domain II of aIF2 (archea) and eIF2 (eukaryotes) but with a high rmsd and a good fit only with α1 (Fig. 2D). Thus, the structure of TipαN34 can be considered as a novel fold containing three regions: a N-terminal flexible extension (NFE), a dodecin-like domain (DOD) and a SAM-like domain (SAM) (Fig. 2C and D).

3.3. Crystal structure of form II and dimer organisation

The structure of crystal form II monomer is essentially identical to crystal form I. Major differences are located in the NFE region that undergoes an important conformational change that swaps the orientation of the N-terminus. Strand β_1 is rotated by 45° and both β2 and β3 are shifted in the structure at pH 4 compared to the crystal form I. Noteworthy, this extension adopts very different conformations in the two chains (Fig. 3A). In chain A, residues from this loop have their side chains oriented towards the solvent, disconnecting the loop from the rest of the molecule and forming a small solvent channel (Fig. 3A). In chain B, the same side chains,

in particular R48, point towards the concave face of the protein which changes the orientation of the loop and closes the solvent channel. The two molecules in the asymmetric unit are arranged in a different dimer than form I (Figs. 1D and 3B). This arrangement also generates a pseudo twofold symmetry axis that goes from the distal N-terminus to the helical bundle burying a total of 1358 Å^2 (Fig. 3B). The two subunits are held together by numerous electrostatic and few hydrophobic interactions in three sites (Fig. 3C). At site 1 the NFEs interact with each other. L37, Q38, D39 and V40 of chain A interact with V40, D39, Q38 and L37 of Chain B, respectively, to form a highly connected structure (Fig. 3C). At site 2, the NFEs interact with each other and the NFE from chain A also contacts β1 of chain B. NFEs interactions include hydrogen bonds between M44 and Q46 and L45 and Q46. In addition, Q46A side chain interacts also with W43 of chain B. On one side only, the side chain of R38 from chain A interacts with D57 from chain B (Fig. 3C). Site 3 comprises interactions between K91 and E92, L95 and N107, S96 and E108, and L95 side chains from both chains.

3.4. Oligomeric state of Tipα

To determine the oligomerisation states of Tipα and TipαN34 in solution, we performed analytical gel filtration experiments (Fig. 3D). At pH 8.5, both Tipα and TipαN34 eluted at a retention volume of 10.9 ml, corresponding to a molecular weight of around 30 kDa, in agreement with a theoretical Tipα homodimer. To mimic the form I crystallisation conditions, we performed analytical gel filtrations at pH 4 and found that TipαN34 eluted at 12 ml, corresponding to a monomer. In contrast, Tipα still eluted as a dimer at around 10.9 ml (Fig. 3D). SDS–PAGE experiments on protein samples corresponding to the elution peak of the two gel filtration experiments, confirm that, in absence of reducing agent, Tipα forms dimers but not TipαN34 (Fig. 3E). This strongly suggests that crystal form I dimer is likely a crystallisation artefact and that TipαN34 dimer observed in crystal from II at pH 8.5 is more likely to be relevant in solution.

4. Conclusions

Tipα is a unique virulence factor, secreted by all *H. pylori* strains, capable of penetrating gastric cells [10]. Our study reveals that this protein adopts an original fold with no resemblance to known PBPs as previously suggested. Tipα structure is a combination of three interconnected regions. These domains point towards putative protein–protein or protein–nucleic acid interaction sites that could play important roles in Tipα function. Finally, C25 and C27 were suggested to be crucial for dimer formation and Tipα activity [10,14]. We have shown that the two cysteines C25 and C27 are not necessary for Tipα to form a dimer at pH 8.5. However, at pH 4, their presence seems to be required in solution to hold the two subunits together. It is possible that *in vivo* these disulfide bridges could be necessary for keeping Tipα in its dimeric form when released into the acidic extracellular medium, i.e., the gastric mucosa.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.04.033.

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